19

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Glazer et al.	Group Art Unit: Not yet assigned
Serial No. 10/617,208	Examiner: Not yet assigned
Filed: July 10, 2003	Attorney Docket No. B00-016-2
For: *Multifunctional Recombinant Phycobiliprotein-Based Fluorescent Constructs and Phycobilisome Display*	

DECLARATION UNDER 37CFR1.131

1. We are the coinventors of the subject patent application.

2. Attached Exhibit IV is photocopies of 15 pages from a laboratory notebook maintained by inventor Yuping Cai during his tenure in the laboratory of inventor Alexander Glazer, and describing our work performed between November 1997 and April 1998 in the United States and which demonstrates our production of (i) a fusion protein comprising a functional displayed domain and a functional phycobiliprotein domain incorporated in a functional oligomeric phycobiliprotein; (ii) a cell comprising a functional oligomeric phycobiliprotein comprising a fusion protein comprising a functional displayed domain and a functional phycobiliprotein domain; and (iii) a fusion protein comprising a functional displayed domain and a functional phycobiliprotein domain incorporated in a functional oligomeric phycobiliprotein, wherein the oligomeric phycobiliprotein provides a fluorescent tag.

3. In particular, the 15 pages are from notebook "YAC #10" section "GCN4pLI Tetramers", and describe experimental result showing the use of Strep-tagged phycocyanin to fluorescently stain streptavidin-coated agarose beads:

Pages 1 to 7: making of expression plasmid pBS323 encoding the HisTag-StrepII-pLI-CpcA fusion protein; and plasmid map and relevant information.

Page 8: purification of the fusion protein from E. coli cells.

Pages 9-15: purification from Anabaena cells, and characterization of An323 (HisTag-StrepII-pLI-CpcA:CpcB recombinant protein); and use of An323 to stain streptavidin-coated agarose beads (page 10). The binding of the StrepII tag-containing recombinant phycobiliprotein An323 to streptavidin was shown to be specific.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

A.N. Glazer 08/02/03

Alexander N. Glazer Date

Yuping Cai Date

Pages 9-15: purification from Anabaena cells, and characterization of An323 (HisTag-StrepII-pLI-CpcA:CpcB recombinant protein); and use of An323 to stain streptavidin-coated agarose beads (page 10). The binding of the StrepII tag-containing recombinant phycobiliprotein An323 to streptavidin was shown to be specific.

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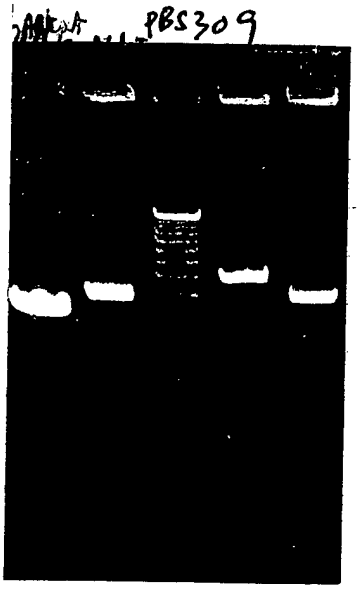
Alexander N. Glazer Date

Yuping Cai 9/2/03 Date

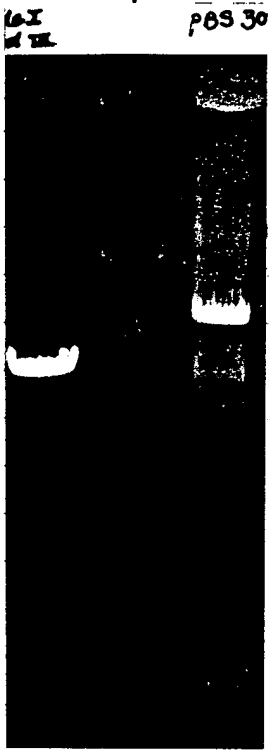
To make HisTag-StrepTag2-GCN4pLI-CpcA:

- pBS309 x NdeI + HindIII ⟶ 4.7
- pANCpcA x NdeI + HindIII ⟶ 0.45



11/21/97. Digestions done by Haidy. Looks like there is a extra NdeI site in pBS309? Need to recheck.

12/10/97. The pBS309 is remade and cleaned up.



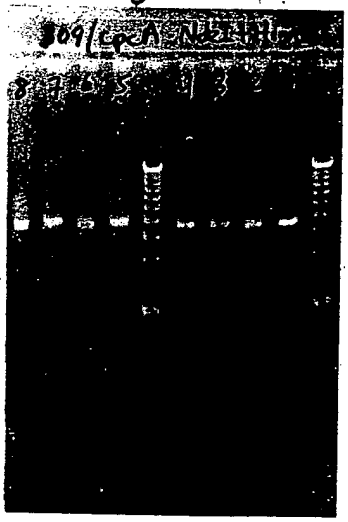
12/18/97. Digestions done by Haidy. Bands cut out, combined, and stored at -20°C.

12/19/97. Gene cleaned and ligated.

12/20/97. Ligation mix used to transform DH5α. Selection: LA + Sp100. 30°C.

12/22/97. About 300 transformants on the 4/10 plate. More on the 9/10 plate. Eight picked for mini-preps.

12/24/97. Mini-preps made by Yuping.

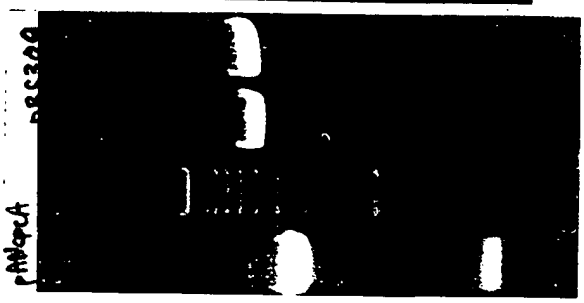


12/26/97. Digestion of the eight mini-preps w/ NdeI + HindIII shows that none has the 0.45-kb insert. Need to repeat.

12/29/97. Digestions repeated. Bands cut out, combined, and stored at -20°C.

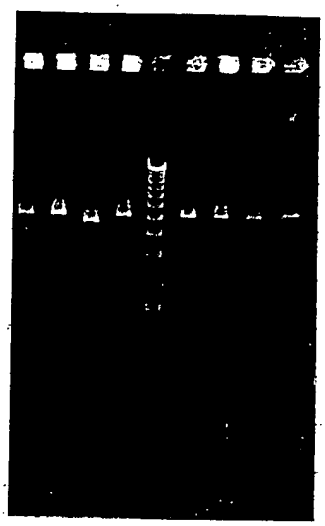
1/7/98. Gene cleaned and ligated.

1/8/98. Ligation mix used to transform DH5α. Selection: LA + Sp. 30°C. Put to R/T the next day.

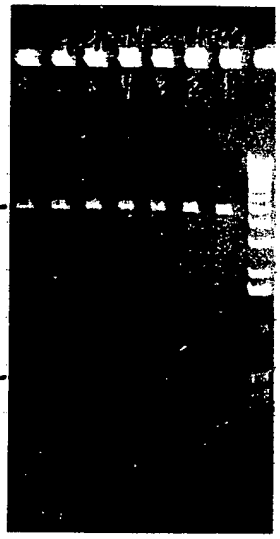


1/12/98. About 200 transformant on the 1/10 plate, more on the 9/10 plate. Eight picked for minipreps.

1/13/98. Minipreps made by Haidy.



1/14/98. Digestion of the eight minipreps (by Steve) with BspLU11I (accidental) shows that #6 doesn't have an insert. The rest need to be check with NdeI + HindIII.

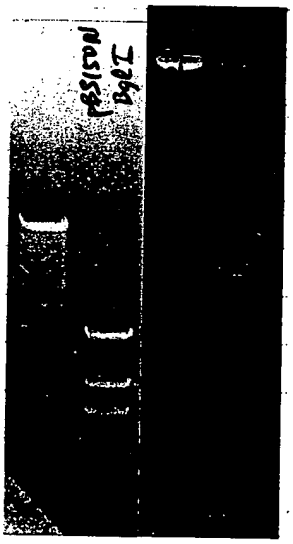


1/15/98. Digestion of the select minipreps (by Haidy) with NdeI + HindIII shows that every one is right.
#4 is saved as pBS323V (5,234 bp)
#4 and #5 used to isolate Ec323.
Both cultures give high yield of the expected 25.5 kD protein. (see gel).

To make pBS323:

pBS323V × BglI → 2625 ←
→ 2609 → EcoRI + HindIII → 2119
→ 490

pBS150N × BglI → 3852 ←
→ 2502 ←
→ 2026



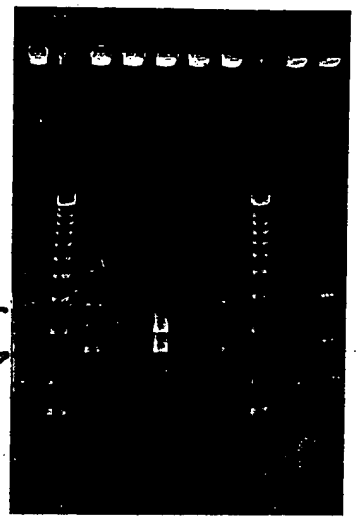
1/16/98. Digestions done. Bands cut out, combined.

1/21/98. Geneclean and ligated.

1/22/98. Ligation mix used to transform DH5α.
Selection: LA + Sp100, 30°C.

1/24/98. About 200 transformants on the 1/10 plate. More on the 9/10 plate. Eight picked for minipreps.

1/27/98. Minipreps made by Wendy.



1/28/98. Digestion of the eight mini-preps (by Steve) with BglI shows that all but #5 look correct.
#7 saved as pBS323 (8,979 bp).

1/29/98. Plasmid DNA of pBS323 used to transform HB101 (pRL528).
Selection: LA + Cm + Sp. 30°C.

1/31/98. Hundreds of transformants obtained

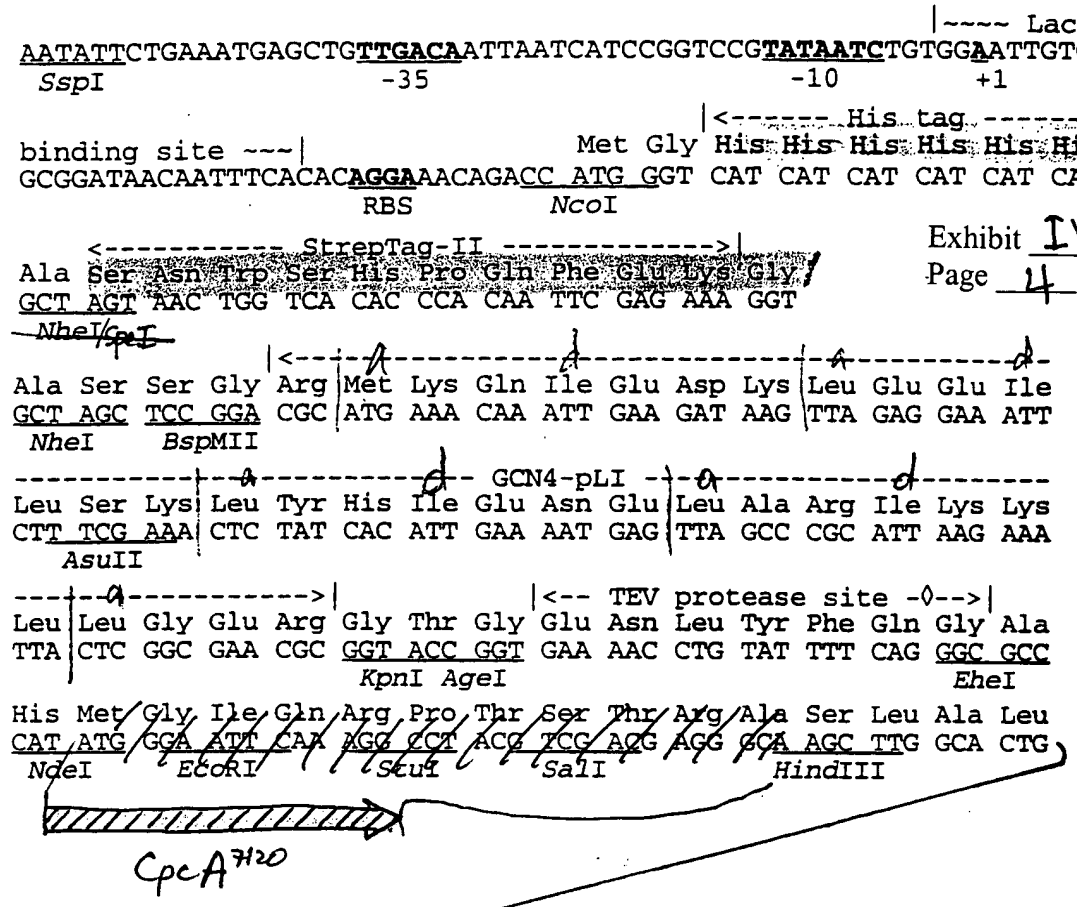
2/14/98. Mating w/ WT7120 performed.

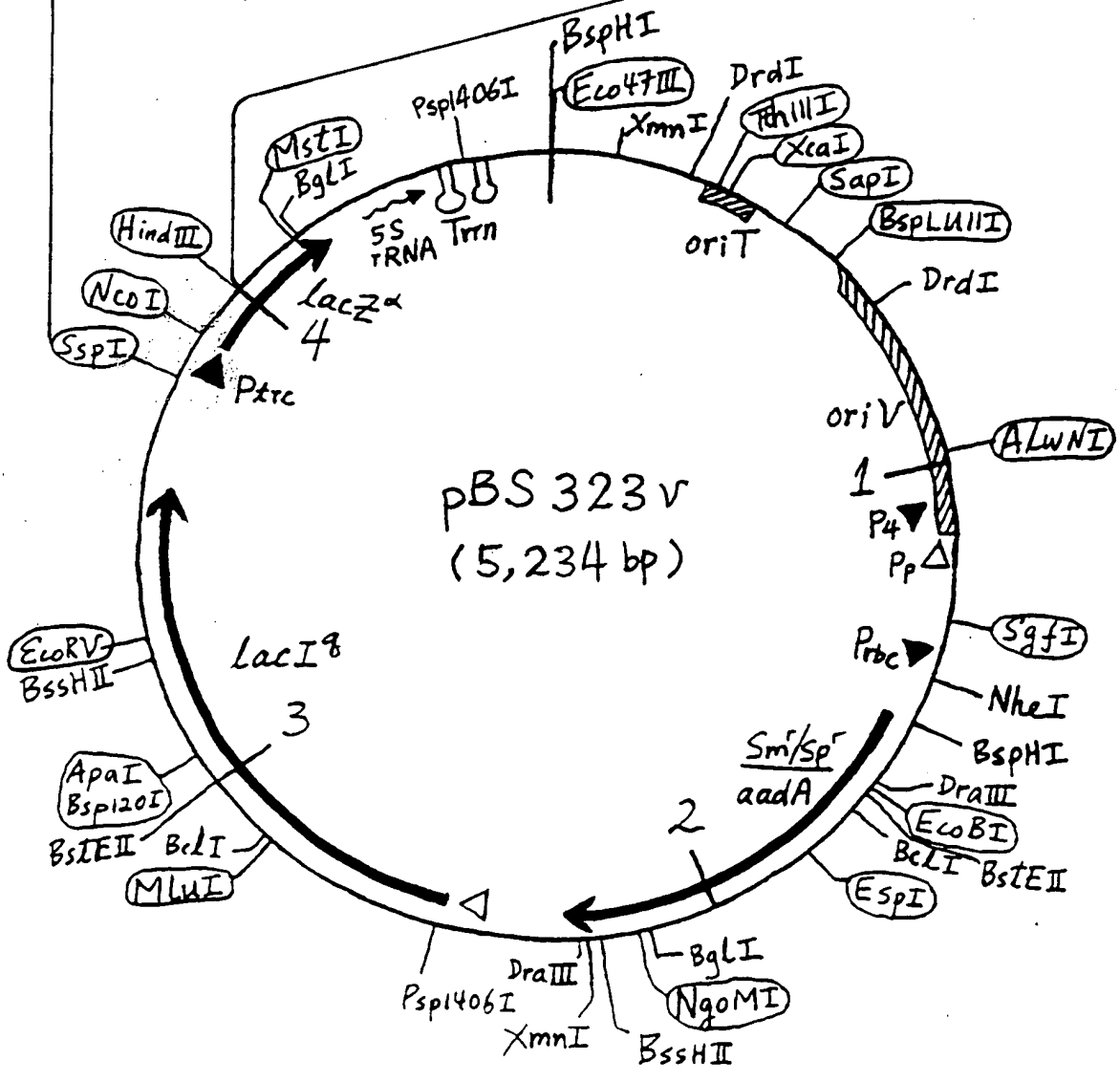
2/14/98. All cell spots have grown some. The mating filter is transferred to AA(N) Sm1.0 Sp10 plate. Put back under HL.

2/19/98. The control spots are dying, and ex-conjugants are seen. The mating filter is transferred to a new plate of AA(N) Sm1.0 Sp10. Put under HL.

2/26/98. Control spots are mostly dead, and lots of exconjugants are seen. Some used to streak on new plates of AA(N) Sm1.0 Sp10, and inoculated AA/8(N) Sp10 flask cultures. Put under HL.

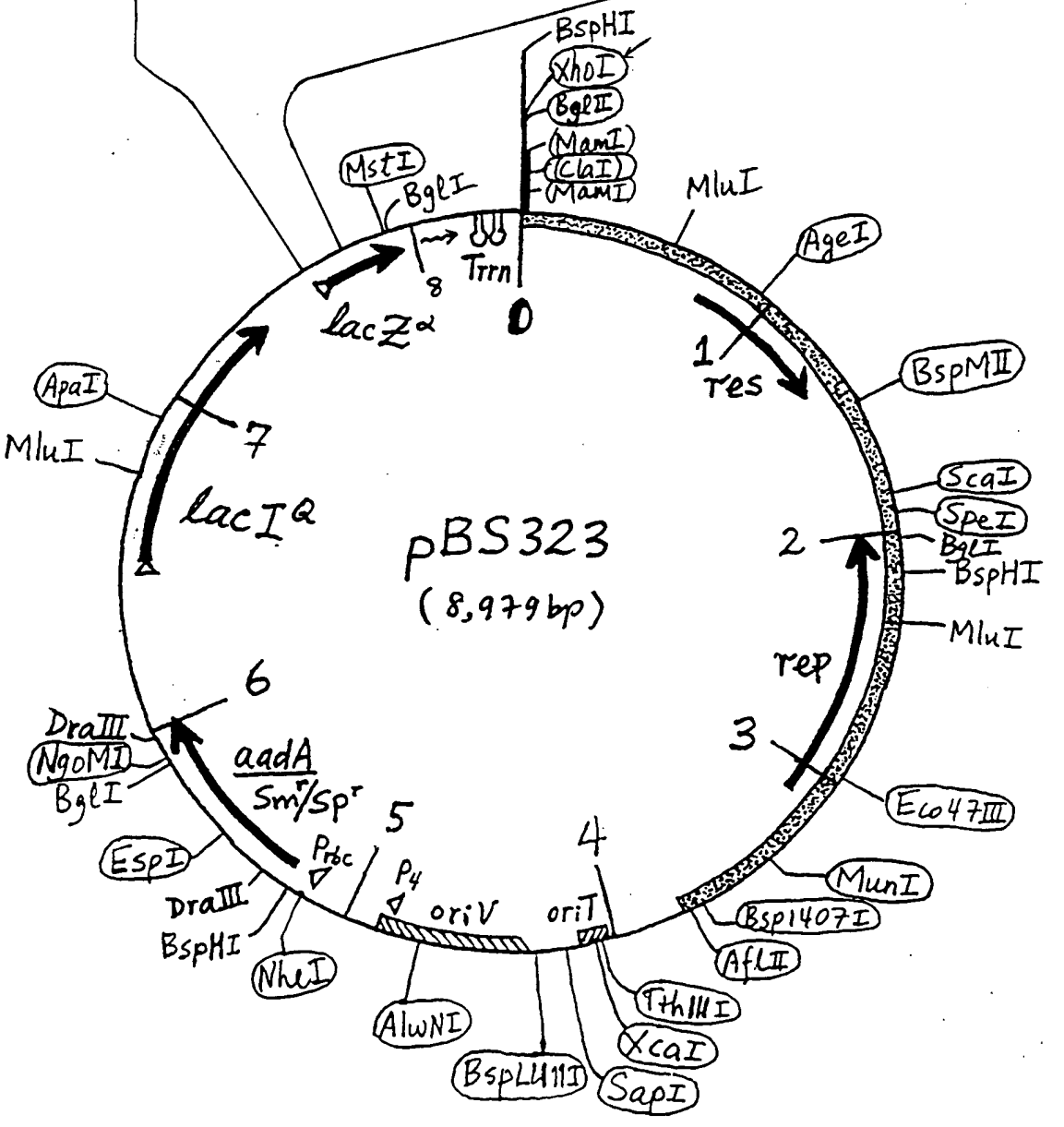
4/17/98. Flask cultures of Anabaena PCC7120 (pBS323) look yellowish and reddish fluorescent. Streaks on plates also look unhealthy.

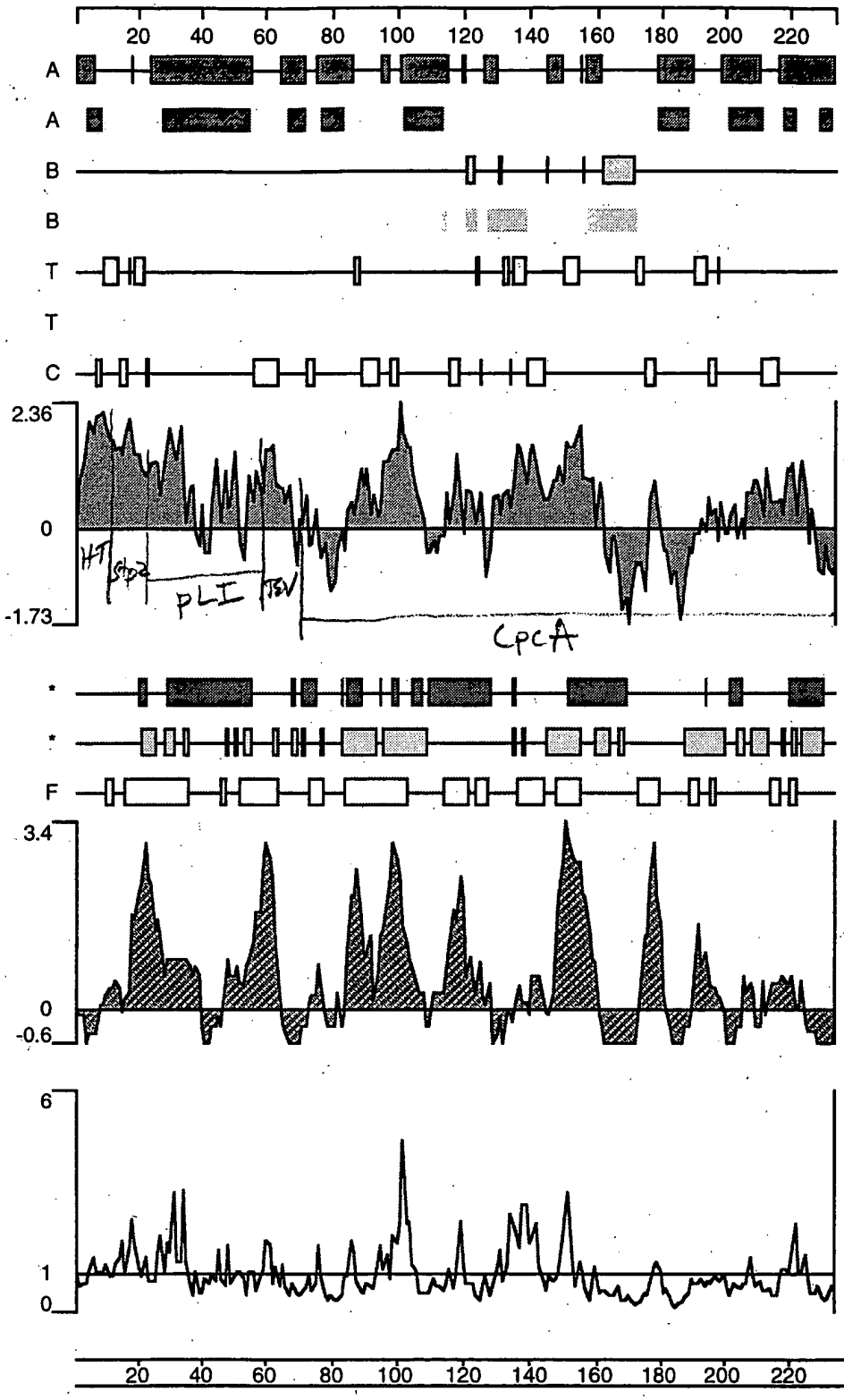

|~~~~ LacIq
AATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGTCCGTATAATCTGTGGAATTGTGA
SspI -35 -10 +1
|<------ His tag ------>|
binding site ~~~| Met Gly His His His His His His
GCGGATAACAATTTCACACAGGAAACAGACC ATG GGT CAT CAT CAT CAT CAT CAC
RBS NcoI
<---------- StrepTag-II ---------->|
Ala Ser Asn Trp Ser His Pro Gln Phe Glu Lys Gly
GCT AGT AAC TGG TCA CAC CCA CAA TTC GAG AAA GGT
NheI/SpeI
Ala Ser Ser Gly Arg Met Lys Gln Ile Glu Asp Lys Leu Glu Glu Ile
GCT AGC TCC GGA CGC ATG AAA CAA ATT GAA GAT AAG TTA GAG GAA ATT
NheI BspMII
Leu Ser Lys Leu Tyr His Ile Glu Asn Glu Leu Ala Arg Ile Lys Lys
CTT TCG AAA CTC TAT CAC ATT GAA AAT GAG TTA GCC CGC ATT AAG AAA
AsuII
GCN4-pLI
Leu Leu Gly Glu Arg Gly Thr Gly Glu Asn Leu Tyr Phe Gln Gly Ala
TTA CTC GGC GAA CGC GGT ACC GGT GAA AAC CTG TAT TTT CAG GGC GCC
KpnI AgeI EheI
<-- TEV protease site -0-->|
His Met Gly Ile Gln Arg Pro Thr Ser Thr Arg Ala Ser Leu Ala Leu
CAT ATG GGA ATT CAA AGG CCT ACG TCG ACG AGG GCA AGC TTG GCA CTG
NdeI EcoRI StuI SalI HindIII
CpcA7H20


pBS323v
(5,234 bp)
BspHI
Eco47III
DrdI
PspI406I
XmnI
Tth111I
XcaI
SapI
BspLU11I
DrdI
MstI
BglI
HindIII
NcoI
SspI
Ptrc
5S rRNA
Trrn
oriT
lacZα 4
oriV
1
ALwNI
P4
Pp
SgfI
Prbc
NheI
BspHI
DraIII
EcoBI
BclI
BstEII
EspI
Sm^r/sp^r aadA
2
BglI
NgoMI
BssHII
XmnI
DraIII
PspI406I
lacIq 3
EcoRV
BssHII
ApaI
Bsp120I
BstEII
BclI
MluI

```
                                                                    |---- LacIq
AATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGTCCGTATAATCTGTGGAATTGTGA
 SspI                    -35                        -10       +1

                                                  |<-------His tag ------>|
binding site ---|                          Met Gly His His His His His His
GCGGATAACAATTTCACACAGGAAACAGACC ATG GGT CAT CAT CAT CAT CAT CAC
                      RBS        NcoI

    <----------- StrepTag-II ------------>|
Ala Ser Asn Trp Ser His Pro Gln Phe Glu Lys Gly
GCT AGT AAC TGG TCA CAC CCA CAA TTC GAG AAA GGT
 NheI/SpeI

                |<----|----------|----------|----------|
Ala Ser Ser Gly Arg|Met Lys Gln Ile Glu Asp Lys|Leu Glu Glu Ile
GCT AGC TCC GGA CGC|ATG AAA CAA ATT GAA GAT AAG|TTA GAG GAA ATT
 NheI   BspMII

----------|----------|-- GCN4-pLI --|----------|--------
Leu Ser Lys|Leu Tyr His Ile Glu Asn Glu|Leu Ala Arg Ile Lys Lys
CTT TCG AAA|CTC TAT CAC ATT GAA AAT GAG|TTA GCC CGC ATT AAG AAA
   AsuII

---|----------->|          |<-- TEV protease site -◊-->|
Leu|Leu Gly Glu Arg Gly Thr Gly Glu Asn Leu Tyr Phe Gln Gly Ala
TTA|CTC GGC GAA CGC GGT ACC GGT GAA AAC CTG TAT TTT CAG GGC GCC
                  KpnI AgeI                          EheI

His Met Gly Ile Gln Arg Pro Thr Ser Thr Arg Ala Ser Leu Ala Leu
CAT ATG GGA ATT CAA AGG CCT ACG TCG ACG AGG GCA AGC TTG GCA CTG
 NdeI  EcoRI  StuI  SalI  HindIII
```

CpcA^{H20}





Alpha, Regions - Garnier-Robson

Beta, Regions - Garnier-Robson

Turn, Regions - Garnier-Robson

Coil, Regions - Garnier-Robson

Hydrophilicity Plot - Kyte-Doolittle

Alpha, Amphipathic Regions - Eisenberg

Beta, Amphipathic Regions - Eisenberg

Flexible Regions - Karplus-Schulz

Antigenic Index - Jameson-Wolf

Surface Probability Plot - Emini

Exhibit IV
Page 7 of 15

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	25525.70 m.w.
Length	233
1 microgram =	39.176 pMoles
Molar Extinction coefficient	25580±5%
1 A(280) =	1.00 mg/ml
Isoelectric Point	7.65
Charge at pH 7	1.90

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	72	38.96	30.90
Acidic (DE)	24	11.70	10.30
Basic (KR)	24	13.37	10.30
Polar (NCQSTY)	64	28.77	27.47
Hydrophobic (AILFWV)	78	30.74	33.48
A Ala	30	8.36	12.88
C Cys	1	0.40	0.43
D Asp	8	3.61	3.43
E Glu	16	8.09	6.87
F Phe	6	3.46	2.58
G Gly	21	4.70	9.01
H His	12	6.45	5.15
I Ile	14	6.21	6.01
K Lys	12	6.03	5.15
L Leu	21	9.31	9.01
M Met	3	1.54	1.29
N Asn	10	4.47	4.29
P Pro	7	2.66	3.00
Q Gln	11	5.52	4.72
R Arg	12	7.34	5.15
S Ser	17	5.80	7.30
T Thr	14	5.54	6.01
V Val	5	1.94	2.15
W Trp	2	1.46	0.86
Y Tyr	11	7.03	4.72
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

323.

25.5 KD

HisTag - StrepTag2 - GCN4pLI - CpcA

25,525.70
+ 585 (PCB)
26,110.70

fully chromophorylated
+ 19,572.00 (β)
45,682.70

EC323V-4
5

5/20/98 by Steve.

Isolation of 6xHis-tagged proteins in GuHCl denaturing condition

(for checking proteins on SDS-PAGE) Cell pellets from ~~0.5~~ 1 liter cultures should be frozen in 400-ml centrifuge tubes at -20°C

Grown up at 30°C, O/N.
IPTG-induced at 37°C for 7 hrs

1. Add the following to solublize cells:
   30 ml **buffer G**
   25 ul **ß-mercaptoethanol** (final ~ 10 mM)
   300 ul 100x **PMSF**
   1 ml 20% **Triton X-100** (final ~ 0.5%)
close bottle and shaker on 30°C shaker for 1 hr.

Exhibit IV
Page 8 of 15

2. Transfer everything to a 40-ml Oakridge tube, balance well, then centrifuge at 15,000 rpm for 20 min at 4°C.

3. Transfer supernate to a new 50-ml tube, add 2 to 3 ml Ni^{2+}-NTA beads suspension, rock in coldroom for 20 min. for His-tagged proteins to bind.

4. Pour in 1.5-cm (diam.) colum, let drain. Reload 2x;

5. Wash with ≥ 10x bed volume **buffer G**;

6. Wash with ≥ 10x bed volume **buffer GA**;

7. Elute with 3 ml **buffer GC**, 2x;
If proteins are to be renatured, sfter step 7 the eluate should be diluted with buffer GA, then dialyze against appropriate buffer (such as 20 mM Tris-HCl pH 8.0, 150 mM NaCl). If the isolated protein contains the core streptavidin (StvC) moiety, the protein needs to be rid of bound biotin by dialysis against 1 to 2 L of 6 M GuHCl pH 1.5 (Urea can not denature StvC completely).

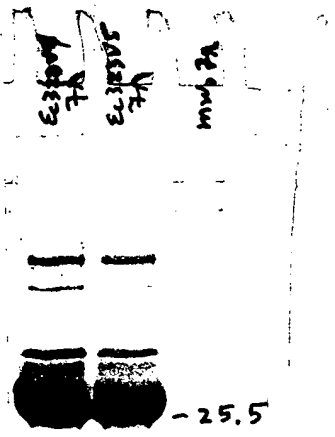
8. Transfer the 6 ml eluate to a dialysis tubing, dialyze against 4 L of H_2O in coldroom for 2 hr to overnight.

Moderate amount of crash outs.

9. If lots of proteins crash out of solution (as expected), retrieve dialysis mixture, spin, dissolve the precipitate with 1x SDS buffer. If not much precipitate, precipitate proteins in the supernate with equal volume of **20% TCA**.

Very little proteins in the supernate (by Spec.).

10 Run SDS-PAGE to check proteins.



Very high yield of the expected 25.5 KD protein!

5/20/98.

An323 (1st)

3/14/98. A 2-L culture of Anabaena PCC7120(pRS323) inoculated from several flask cultures (washed 2x), into AA⑧/2 + 3 mM HEPES pH + Sp20. Put under HL, bubbled.

3/19/98. The culture has grown to a moderately dense one. relatively healthy-looking. yellowish green. DPTC added to final 0.5 mM. Put back under HL. bubbled.

3/23/98. The culture has grown to a near black one. whole-cell spec: 80323402.sp. Cells harvested by centrifugation — supernate medium is light yellowish green. Wet cell weight = 1.6 + 1.7 + 2.2 gm. Stored at −20°C

3/27/98. The 2.2 gm cell pellet used to make An323 protein. (by may/Steve/Yuping. see attached sheets).

12.5 ml total after dialysis.

$$A_{622} = A_{623} = 0.1933 \times 10 = 1.933$$

$$[\text{mg/ml}] = \frac{A}{\varepsilon} \cdot MW$$

$$= \frac{1.933}{290{,}000} \times 45{,}682.70$$

$$= \underline{0.3 \text{ mg/ml}}$$

$$[M] = \frac{1.933}{290{,}000} = \underline{6.7\,\mu M}$$

4/2/98. Use StvC-Agarose beads to test binding.

StvC-Agarose beads from Sigma:

50% suspension. Specification: 1 ml packed gel binds 23 µg Biotin.

MW Biotin = 244.31; 23 µg ⇒ 0.09414 µMole
= 94.14 n'Mole

So: 2 ml bead suspension = 1 ml packed beads have 23.5 nMole immobilized StvC.

⇒ 2.35 nMole StvC/200 µl bead suspension.

≈ 1.2 nMole StvC/100 µl bead suspension.

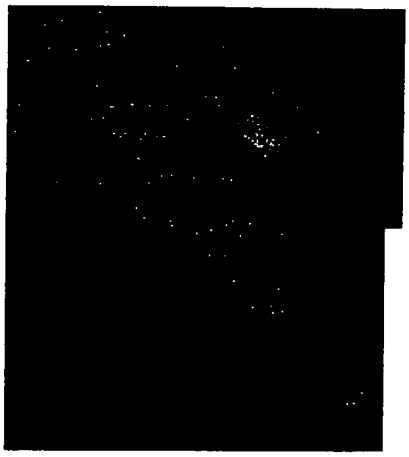
≈ 4.7 nMole binding site/100 µl bead suspension.

An323: 6.7 n Mole monomer/ml,
1.4 ml ⇒ 9.4 nMole monomer.
⇒ 2:1 binding to 100 µl Bead suspension

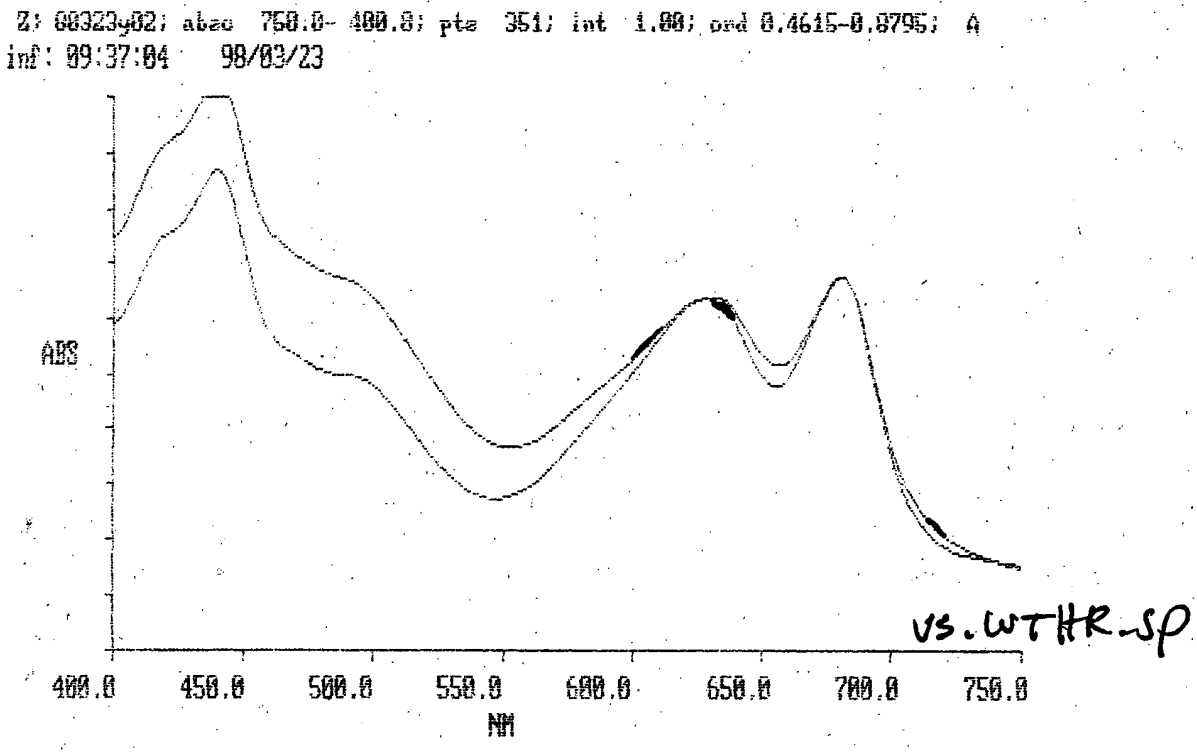
An321: 8.83 n Mole monomer/ml.
1.064 ml ⇒ 9.4 nMole monomer
⇒ 2:1 binding to 100 µl Beads suspension as control.

Beads washed w/ buffer Φ 2x.
look under UV: An323 beads much more fluorescent than An321.

To be sure of specific Binding. Beads washed 2x w/ 100 µM Biotin in buffer Φ. Then look at UV: Most color on An323 beads gone!
⟹ Specific binding of An323 to StvC!



4/30/98. Use StvC-coated beads from Biometra.
(Recombinant StvC may work better).
Experiment repeated as above. got similar but better results (binding of An323 gave better/stronger fluorescence).



— Anabaena PCC7120 (pBS323)

2-L culture in AA(N)/2 + 3 mM HEPES pH 9.0 + Sp20. Grown under Ht. bubbled.

IPTG-induced for 3.5 days. under Ht. bubbled.

An323 2.279 from 3/25/96 by May & Steve 3/30/96

HisTag-StrepTag2-pLI-CpcA

Isolation of HisTagged proteins from *Anabaena* PCC7120

(This method gets **upper limit** on yield; This is for using cells with wet weight ranging from **1 to 2 grams**. It may be necessary to scale down or up the prep with different amount of cells)

Before starting: reserve the Ti60 angled rotor from Nikaido lab and cool it in the coldroom, and reserve the Ultracentrifuge; make sure that there are as many 23-ml ultra-centrifuge tubes as the number of samples; should have all the buffers ice-cold; should not do more than eight samples at a time.

1. Thaw frozen cell pellet on ice, add ice-cold **buffer 0** to final 18 ml. Vortex to resuspend and homogenize.

2. Add (immediately before French press):
   12 µl pure **β-mercaptoethanol** (final 10 mM)
   200 µl 100x **PMSF stock** (final 1mM)
Invert several times to mix.

3. Transfer cell suspension to the large French Press cell; pass 3x at 18,000 psi ("high" setting dial to 1135); cool on ice afterwards.

4. Transfer cell lysate to a 23-ml ultracentrifuge tube, balance well, spin at 37,000 rpm (130,000 x *g*) at 4°C for 60 min. in the Ti60 rotor. Promptly take out the tubes after c ntrifuge.

5. Carefully pour out the supernate to a new 50-ml tube ---- take care not to carry over any membrane fraction. (the cell lysate supernate volume should be about 20 ml)

filename: 80320504

6. Bring up volume to 20 ml with **buffer 0**; spec: 50x

7. Use 1 to 2 ml of Ni^{2+}-NTA beads suspension to set up column, use of 1.0-cm diam. column is preferred.

8. Carefully pour lysate supernate into column, let settle a bit, then let drip slowly into a new 50-ml tube.

9. Reload flow-thru lysate supernate on the column, let drip slowly.

10. (Resuspend beads in all the following washes):
    Wash with 1 column-full (about 20 ml) of **buffer A1**;
    Wash with 1 column-full of **buffer B**;
    Wash with 1 column-full of **buffer A2**.

Color of the beads after washing: dark/deep blue
Beads overloaded? likely

11. Elute HisTagged proteins with 2 ml of eluent **buffer C** (resuspend beads), twice; [elute with another 2 ml of **buffer C** if necessary (do not resuspend beads, and let drip slowly].

Color of the eluate: clear deep blue

fairly hard to elute.
6 x 2ml total.
Beads still ~~quite~~ blue.

13. If the eluate has color, spec from 350 to 750 nm (can not spec with shorter wavelengths because of imidazole).

14. Immediately dialyze samples to eliminate imidazole ---- the 200 mM imidazole in **buffer C** denatures proteins slightly, and should not be with the proteins for an extended period of time.

Usual dialysis conditions (all should be done at 4°C):
(all need 50 ml of 2 M Tris HCl pH 8.0, 50 ml of 4 M Na/KCl, and 1 ml of 1 M DTT)

4 liters of 25 mM Tris pH 8.0, 50 mM Na/KCl, 0.5 mM DTT; o/N

change buffer 1 ~~or 2~~ times (depending on number of samples); then

2 liters of 50 mM Tris pH 8.0, 100 mM Na/KCl, 1 mM DTT.

15. Retrieve sample, and spec (use the final dialysis buffer to background).

12.5 ml; some precipitate.

All buffers should be kept at 4°C:

Buffer 0:
20 mM Tris HCl pH 8.0
100 mM Na/KCl

To make 1 liter: Add to 965 ml of MQ-H_2O
10 ml of 2 M Tris HCl pH 8.0
25 ml of 4 M Na/KCl

Buffer A1:
20 mM Tris HCl pH 8.0
100 mM Na/KCl
20 mM imidazole
5% glycerol

To make 1 liter: Add to 898 ml of MQ-H_2O
10 ml of 2 M Tris HCl pH 8.0
25 ml of 4 M Na/KCl
4 ml of 5 M imidazole
63 ml of 80% glycerol

Buffer B:
20 mM Tris HCl pH 8.0
1 M (!) Na/KCl

To make 1 liter: Add to 740 ml of MQ-H_2O
10 ml of 2 M Tris HCl pH 8.0
250 ml of 4 M Na/KCl

Buffer A2:
20 mM Tris HCl pH 8.0
100 mM Na/KCl
30 mM imidazole

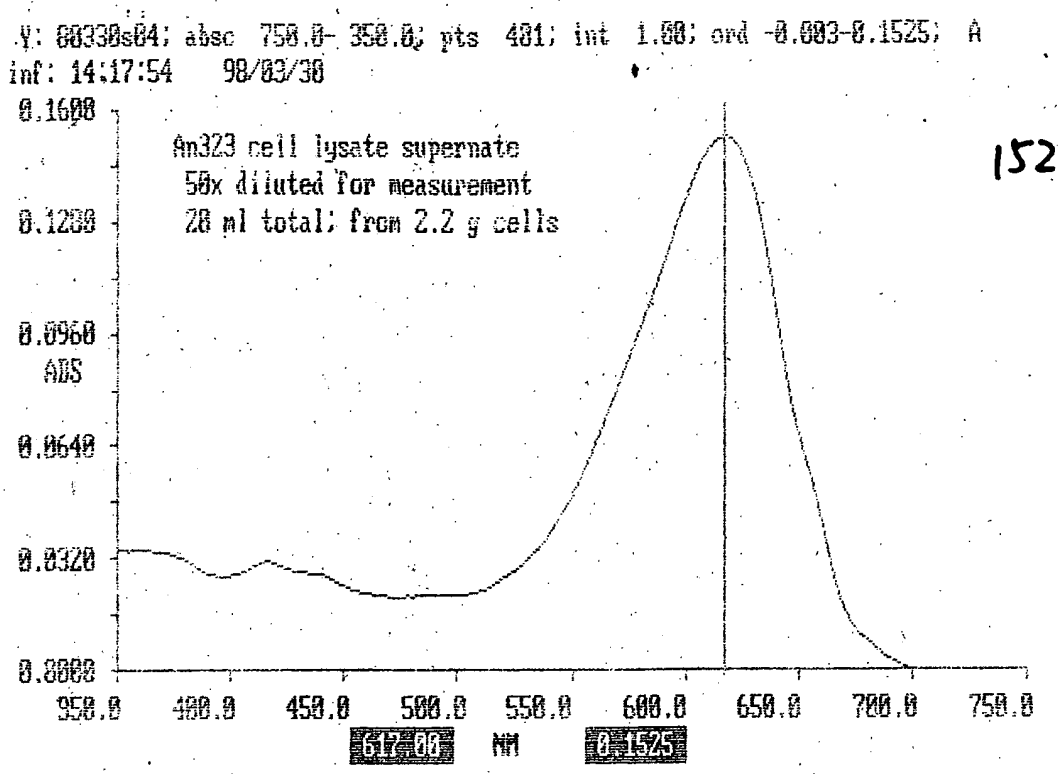
To make 1 liter: Add to 959 ml of MQ-H_2O
10 ml of 2 M Tris HCl pH 8.0
25 ml of 4 M Na/KCl
6 ml of 5 M imidazole

Buffer C:
20 mM Tris HCl pH 8.0
100 mM Na/KCl
200 mM imidazole

To make 0.5 liter: Add to 462.5 ml of MQ-H_2O
5 ml of 2 M Tris HCl pH 8.0
12.5 ml of 4 M Na/KCl
20 ml of 5 M imidazole

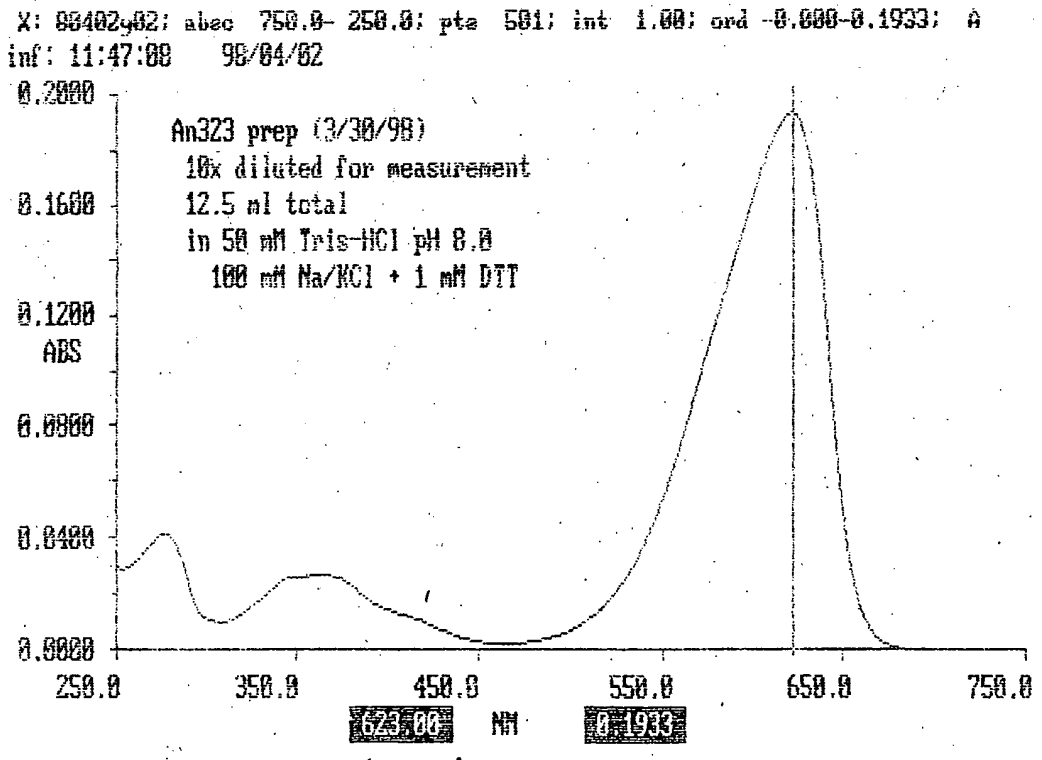
Other stock solutions needed:

100 mM PMSF (Dissolve ~~1.74~~ 0.174g grams of PhenylMethylSulfonyl Fluoride in 10mL pure EtOH)
5 M imidazole (keep at 4°C)
4 M Na/KCl (2 M NaCl + 2 M KCl)
2 M Tris HCl pH 8.0
2 M Tris HCl pH 7.0



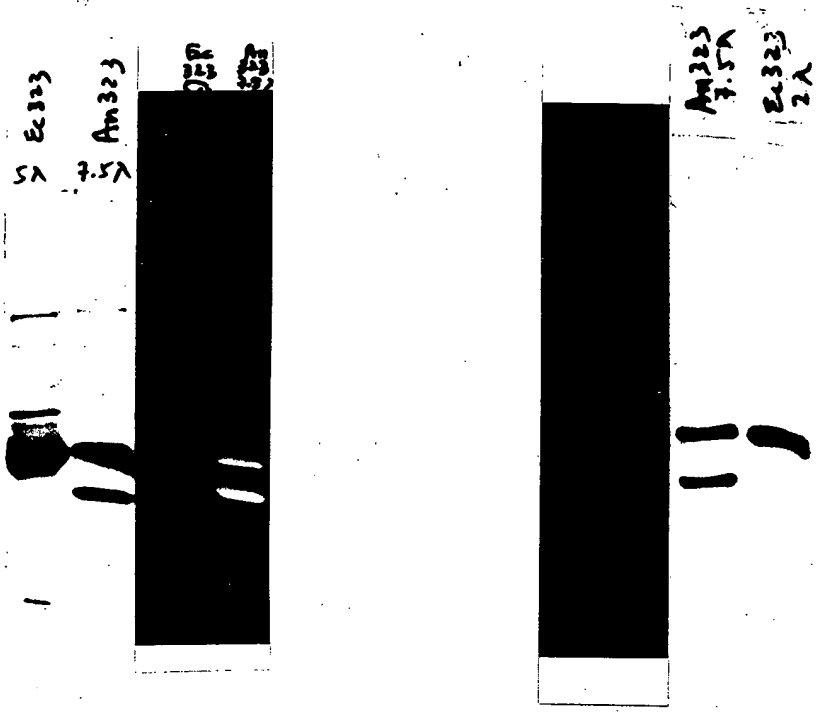
152.5 $A_{617} \cdot ml$

Beads overload
non-elutable pigm'ts. ≥15.8%



24.2 $A_{623} \cdot ml$

622 abs


Eλ323
5λ
Aλ323
7.5λ
Aλ323
7.5λ
Eλ323
2λ